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Cellular and Humoral Immune Responses in Dogs Experimentally and Naturally Infected with *Leishmania infantum*

ELENA PINELLI,[1]* ROBERT KILLICK-KENDRICK,[2] JOSÉE WAGENAAR,[1] WILBERT BERNADINA,[1] GUSTAVO DEL REAL,[3] AND JOOST RUITENBERG[1,4]

Department of Immunology, Faculty of Veterinary Medicine, 3584 CL Utrecht,[1] and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam,[4] The Netherlands; Department of Biology, Imperial College at Silwood Park, Ascot, Berks SL5 7PY, United Kingdom[2]; and Instituto Llorente, 28035 Madrid, Spain[3]

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In this paper we describe a number of immunological parameters for dogs with a chronic *Leishmania infantum* infection which exhibit patterns of progressive disease or apparent resistance. The outcome of infection was assessed by isolation of parasites, serum antibody titers to *Leishmania* antigen, and development of clinical signs of leishmaniasis. Our studies show that 3 years after experimental infection, asymptomatic or resistant dogs responded to *L. infantum* antigen both in lymphocyte proliferation assays in vitro and in delayed-type hypersensitivity reaction, whereas no serum antibodies to parasite antigen were shown. In contrast, symptomatic or susceptible animals failed to respond to parasite antigen in cell-mediated assays both in vitro and in vivo and showed considerably higher serum antibodies to leishmanial antigens. In addition, significantly higher level of interleukin 2 (IL-2) and tumor necrosis factor were found in supernatants from stimulated peripheral mononuclear cells from asymptomatic dogs compared with those from symptomatic and control uninfected dogs. IL-6 production did not vary significantly among the groups studied. Finally, we observed similar results with a group of mixed-breed dogs with natural *Leishmania* infections also grouped as asymptomatic or symptomatic on the basis of clinical signs of canine visceral leishmaniasis. These results demonstrate that serum antibody titers, antigen-specific proliferative responses, delayed-type hypersensitivity skin reactions, and IL-2 and tumor necrosis factor production by peripheral mononuclear cells can be used as markers of disease progression.

Visceral leishmaniasis (VL) is a disease which, in the Mediterranean countries, affects both humans (3, 13, 22, 26) and dogs (1, 10, 12, 17, 34). The disease is caused by *Leishmania infantum* parasites (29) that are transmitted to vertebrate hosts by sand fly vectors of the genus *Phlebotomus*. After multiplication within macrophages at the site of infection, the parasites may leave the skin and spread to mononuclear phagocytes of the reticuloendothelial system, including the spleen, liver, and bone marrow, to cause a chronic, sometimes fatal, disease.

Studies with mouse models have demonstrated that the generation of protective immunity against leishmaniasis is T cell and cytokine mediated (8, 19, 33, 35). Thus, the absence of such immune responses has been associated with chronicity of the disease and high mortality in untreated cases (6, 30). However, studies on the protective role of T cells (2, 4, 5) and cytokines in natural host models of VL, such as the dog, are limited. Moreover, attempts to immunize dogs against VL according to immunization procedures conferring immunity to *Leishmania* infection in mice have led to exacerbation of the disease (9). The increased susceptibility to the parasite in such vaccination trials may be the result of the differences existing between natural hosts of *Leishmania* and experimental animal substitutes. It is therefore necessary to conduct further studies on canine VL (CVL) in order to identify the mechanisms underlying either resistance or susceptibility to *Leishmania* infection in this species.

In a recent study on resistance to *L. infantum* in trial-vaccinated beagles (27), we observed that at 3 years post-challenge, the study population comprised asymptomatic, apparently resistant dogs and symptomatic animals susceptible to the experimental infection. We made an identical observation with a group of mixed-breed dogs from the Madrid area with natural *Leishmania* infections. The availability of dogs either resistant or susceptible to *L. infantum* prompted us to examine the roles of antibodies, T cells, and cytokines in *Leishmania* infections in these dogs.

In this paper we describe the humoral and cellular immune status of dogs with a chronic infection manifesting either resistance or susceptibility to *L. infantum* after experimental or natural infection with this parasite. In addition, we present the profile of a number of interleukins (interleukin 2 [IL-2], IL-6, and tumor necrosis factor [TNF]) known to be involved in the immune response to protozoan parasites as well as some preliminary markers of CVL emerging from this study.

MATERIALS AND METHODS

Animals. Three groups of dogs were used as sources of peripheral blood mononuclear cells (PBMC) in this study. The first comprised dogs selected from a colony of beagles bred and maintained under conditions designed to exclude any possible contaminating *Leishmania* infections, at the Barajas

* Corresponding author. Mailing address: Department of Immunology, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 1, P.O. Box 80.165, 3508 TD Utrecht, The Netherlands. Phone: 31-30-534345. Fax: 31-30-533555.

(Madrid) animal facility of the Llorente Institute. The group of beagles included all dogs previously exposed to *L. infantum* infections in a vaccine development program (27).

The second group consisted of dogs with natural *L. infantum* infections. This group of mixed-breed dogs was housed at an animal shelter run by volunteers in Alcala de Henares (Spain).

The third group comprised beagles (designated nonendemic controls) housed at the Utrecht (The Netherlands) Faculty of Veterinary Medicine animal facility. Group 1 and 3 dogs were well-fed animals under constant scrutiny for health problems by a veterinarian and had all received their yearly routine vaccinations against leptospirosis, distemper, adenovirosis, parainfluenza, and parvovirosis.

Experimental infections and isolation of parasites. The procedures followed to produce *L. infantum* infections in the Barajas colony of beagles will be described in greater detail in a separate report. Briefly, dogs were inoculated with promastigotes of *L. infantum* harvested from the midguts of experimentally infected *Phlebotomus perniciosus*. The sand flies were the second generation of a laboratory colony set up in July 1988 with female flies collected near Murcia, Spain. The flies were infected by permitting them to feed on the head of a tranquilized dog with leishmaniasis contracted in the Madrid area, where the disease is endemic. They were kept at 26°C in high humidity and were given fresh sucrose solution daily. Ten days later, flies were dissected and promastigotes were harvested from the midgut. Each dog was given 8×10^4 promastigotes intradermally, in two doses (0.1 ml each). One dose contained half the contents of one salivary gland of *P. perniciosus*, and the other did not. Two hours after the last dog was inoculated, four hamsters were given intradermal inoculations of the same suspensions given to the dogs. The hamsters served as controls for the infectivity of the parasites. Infection of the dogs was assessed by collecting bone marrow aspirates and spleen tissue biopsies followed by culturing of parasites in NNN biphasic medium.

L. infantum parasites (MCAN/ES/88/1SS441 DOBA) were expanded at 25°C in Schneider's insect culture medium (GIBCO, Paisley, Renfrewshire, United Kingdom) supplemented with 10% fetal calf serum (Sera Lab, Crawley Down, Sussex, United Kingdom), 100 IU of penicillin (GIBCO) per ml, 100 μg of streptomycin (GIBCO) per ml, and 2 mM L-glutamine (GIBCO). When at stationary phase, promastigotes were harvested, washed, and used for routine serological surveillance of canine leishmaniasis and for preparing *Leishmania* soluble antigen (LSA).

Assessment of susceptibility or resistance to infection. Infected dogs were monitored for parasite establishment and subsequently for the development of leishmaniasis. This was achieved by routine screening of the dogs for classical clinical signs such as skin lesions, alopecia, popliteal lymph node size, weight loss, skin ulceration, excessive nail growth, and parasite isolation. Initial clinical signs of CVL were observed 18 months following inoculation. At 3 years after experimental infection, dogs were grouped as *L. infantum*-susceptible or -resistant animals on the basis of the positive or negative outcome of clinical parameters.

Serum anti-*Leishmania* antibodies (indicating parasite establishment and possible disease development) were assayed by a standard indirect immunofluorescence assay (IFA). Briefly, serum dilutions in phosphate-buffered (0.07 M) saline (0.15 M) (PBS; pH 7.2) were added to 5×10^4 cultured *L. infantum* promastigotes (air dried and acetone fixed on glass slides) and incubated at 37°C for 1 h in a moist atmosphere. After washes with PBS, a rabbit anti-dog immunoglobulin G heavy plus light chain conjugated with fluorescein isothiocyanate (Nordic Immunology, Cultek S.L., Madrid, Spain) was added to the slides. Following incubation for a further hour at 37°C and washes with PBS, the slides were scored for IFA titers by using a fluorescence microscope (i.e., the highest serum dilution still giving a positive IFA score compared with that for a negative control serum). IFA titers greater than 1:80 were considered positive.

Antigens and mitogen. LSA and Dohyvac (Duphar B.V., Weesp, The Netherlands), a canine vaccine against distemper, hepatitis, and parainfluenza, were used as stimulator antigens in lymphocyte proliferation assays and long-term lymphocyte cultures. LSA was prepared by the following procedure. Washed, cultured *L. infantum* promastigotes at a concentration of 2×10^8/ml in PBS were frozen and thawed three times and then centrifuged (8,000 × *g*, 30 min, 4°C), and the resulting supernatants were collected. Protein concentration of the supernatants was determined by the Lowry technique (21), and aliquots (1 mg/ml) of LSA were stored at −20°C until use. Concanavalin A (ConA) purchased from Sigma Chemical Co. (St. Louis, Mo.) was used as a T-cell mitogen.

Lymphocyte proliferation assay. PBMC were prepared from heparinized blood by centrifugation over Ficoll (density, 1.078) for 25 min at 800 × *g* at room temperature. Cells were cultured in flat-bottom 96-well microtiter plates (Costar, Cambridge, Mass.) at a density of 2×10^5 cells per well in the presence of 10 μg of LSA per ml, 2 μg of ConA per ml, and 35 μg of Dohyvac vaccine per ml or without antigen in a total volume of 200 μl of RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 10^{-5} M 2-mercaptoethanol (Fluka AG, Buchs, Switzerland), hereafter designated complete medium. Optimal concentrations of antigens and mitogen were determined in kinetic experiments performed prior to the present study. PBMC were incubated for 5 days in a humidified atmosphere at 37°C and 5% CO_2 and pulsed during the last 18 h with 0.4 μCi of [^{3}H]thymidine (1.0 Ci/mmol; Amersham, Bucks, United Kingdom). Cells were harvested onto glass fiber filters, and [^{3}H]thymidine incorporation was determined by liquid scintillation counting. All tests were performed in triplicate. Proliferative responses were expressed as the stimulation indices (SI), which represent the ratio of mean proliferation after stimulation to the mean proliferation of medium controls.

In vitro cytokine production and determination. Cytokine production by PBMC was evaluated as follows. PBMC at 2×10^6 cells per ml in complete medium were cultured in the presence of LSA (10 μg/ml) or ConA (2.5 μg/ml). After 48 h of culture, cells were centrifuged (500 × *g*, 10 min), the resulting supernatant was filtered (0.2-μm-pore-size filter; Millipore S.A., Molsheim, France), and the samples were stored at −20°C until use. Culture supernatants were tested for IL-6 activity with the B9 assay as described elsewhere (15). Briefly, murine hybridoma cells (5×10^3 cells per well) were cultured in 96-well flat-bottom microtiter plates in RPMI 1640 supplemented with 10% fetal calf serum. Test samples were titrated in twofold dilutions. The IL-6 concentration was determined by comparing test sample data with those obtained with the recombinant human IL-6 used as a standard. After 64 h at 37°C and 5% CO_2, cells were pulsed during the last 18 h of culture with [^{3}H]thymidine. Cells were

harvested onto glass fiber filters, and [^{3}H]thymidine incorporation was measured. The recombinant human IL-6 (specific activity, 10^6 U/μg) purified from *Escherichia coli* was a kind gift from L. A. Aarden, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

IL-2 activity was determined by modification of a bioassay described elsewhere (28). Long-term cultures of canine IL-2-dependent T-cell blasts were generated by stimulation of PBMC (2×10^6 cells per ml) with 2.5 μg of ConA per ml for 6 days at 37°C and 5% CO_2. Cells (2×10^4 per well) were cultured for 48 h with test samples in twofold dilution and pulsed with [^{3}H]thymidine as described above. The IL-2 concentration was estimated by using recombinant human IL-2 as a standard (specific activity, 18.8×10^6 U/mg). Human recombinant IL-2, purified from Chinese hamster ovary cells, transfected with the human IL-2 gene was kindly provided by Sanofi, Toulouse, France.

TNF activity was determined by modification of an in vitro cytotoxicity bioassay, with WEHI 164 murine fibrosarcoma cells (11). Briefly, 2×10^4 cells per well in RPMI 1640 medium supplemented with 10% fetal calf serum were cultured for 24 h with test samples in twofold dilutions in a 100-μl total volume. Cell survival was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) colorimetric assay (24). TNF concentration was estimated by using murine recombinant TNF-α (Genzyme, Cambridge, Mass.) as a standard (specific activity, 4×10^7 U/mg).

Statistical analysis. Correlation between cytokine production by canine PBMC and resistance or susceptibility to *Leishmania* infection was evaluated by using Student's *t* test. Significance was considered at *P* values of <0.05.

DTH reactions. Dogs were tested for delayed-type hypersensitivity (DTH) reaction to leishmanin by intradermal injection of 0.1 ml of leishmanin reagent (inactivated suspension of 3×10^8 *L. infantum* promastigotes per ml in 0.01% merthiolate-saline; Llorente S.A., Madrid, Spain). Skin reactions were recorded after 48 h, and an induration or redness area of >5 mm in diameter was considered positive. Purified protein derivative (Llorente) and leishmanin diluent as well as saline solution (0.1 ml of each) served as controls.

RESULTS

Leishmania parasites and antileishmanial antibodies in the Barajas colony of beagles. The presence in the Barajas colony of dogs of *L. infantum* infections that were restricted to animals previously infected was reconfirmed in this study by isolation of parasites from such animals only (Table 1). Parasites were isolated from 10 of 12 infected dogs. Furthermore, antileishmanial antibodies were absent in previously noninfected animals, as were clinical features of leishmaniasis, whereas both antileishmanial antibodies and clinical signs of disease were observed only in previously infected dogs.

Lymphocyte proliferative responses. Table 2 shows the lymphocyte proliferative responses of PBMC to parasite antigen and mitogen in the three groups of animals. Cells from infected asymptomatic dogs responded to LSA with a SI ranging from 4.1 to 160. In contrast, when cells from symptomatic dogs were cultured in the presence of LSA, they gave negative test responses, similar to test responses obtained with PBMC from control uninfected dogs. The SI in each case was less than 2, with absolute responses of less than 300 cpm. Lymphocyte proliferation to ConA was high in all dogs analyzed and showed considerable variation. Cells from symptomatic dogs responded to ConA in a manner similar to those from both the control and the asymptomatic groups of dogs. When test responses are expressed as SI values, in the latter group four of eight dogs showed higher proliferative responses to ConA compared with all other animals studied. The absolute response in counts per minute was nevertheless comparable among the three groups. It was noted that a similar pattern of proliferative responses to LSA and ConA occurred when cells from mixed-breed dogs with natural *Leishmania* infections were tested by lymphocyte proliferative assay: asymptomatic dogs show higher proliferative responses to LSA compared with those in symptomatic dogs.

A possible explanation for the observed weak proliferative test responses to LSA in this study is that in dogs affected by leishmaniasis, responses to all antigens are depressed. Therefore, we examined responses to a positive control antigen, namely, Dohyvac, a vaccine that is routinely administered to the dogs studied here. By doing so, a positive, though variable, proliferative response occurred in all dogs studied (Table 2). This indicates that depressed reactivity to LSA in *Leishmania*-susceptible dogs was antigen specific.

DTH skin reaction to leishmanin. The extents of DTH skin reactions to leishmanin in the Barajas groups of dogs are presented in Table 3. Asymptomatic dogs reacted positively to intradermal injection of leishmanin. Positive responses were found in six of seven dogs tested, whereas all control uninfected dogs and two of three symptomatic animals did not respond. Dogs were also tested for their reactivity to

TABLE 1. Clinical features of leishmaniasis, antiparasite antibody titers, and parasite isolation from dogs selected from the Barajas colony

Infection status[a] and dog	Clinical feature[b]	Antiparasite antibodies[c]	Parasite isolation
NI			
1	−	<10	No
7	−	<10	No
9	−	<10	No
27	−	<10	No
32	−	<10	No
38	−	<10	No
50	−	<10	No
60	−	<10	No
63	−	<10	No
67	−	<10	No
I			
2	−	<10	Yes
12	−	<10	No
14	++	320	Yes
16	−	80	Yes
17	++	640	Yes
19	+++	320	Yes
20	−	20	Yes
21	+++	640	Yes
23	−	<10	No
28	−	<10	Yes
30	−	<10	Yes
44	−	<10	Yes

[a] NI, noninfected; I, experimentally infected beagles.
[b] Clinical features of leishmaniasis were scored on the bases of weight loss (+); alopecia, increased popliteal lymph nodes, and weight loss (++); or excessive nail growth, skin lesions, and the other previously described clinical signs (+++). −, absence of clinical signs.
[c] Values are expressed as the reciprocals of IFA titers.

TABLE 2. Proliferative responses of PBMC from infected as well as from uninfected dogs

Group and dog	No antigen (cpm)	Lymphocyte proliferation (SI) to:		
		ConA	LSA	Dohyvac
Control uninfected				
4	67	225	<2.0	2.0
9	62	437	<2.0	ND[a]
58	122	133	<2.0	ND
59	57	201	<2.0	2.0
919	84	274	<2.0	3.1
154	99	98	<2.0	5.4
155	89	170	<2.0	2.0
158	122	160	<2.0	5.2
Infected				
Asymptomatic or resistant				
2	39	621	160	4.0
12	116	458	32	2.0
16	54	686	23	2.6
20	63	321	5.7	<2.0
23	30	589	7.5	3.1
28	95	125	47	6.2
30	126	171	6.3	ND
44	42	341	4.6	11.7
MB 1[b]	89	494	4.1	3.1
MB 2	30	672	96	3.2
MB 3	60	249	12	2.2
Symptomatic or susceptible				
17	70	173	<2.0	2.6
19	72	179	<2.0	<2.0
21	128	254	<2.0	ND
MB 4	21	214	<2.0	2.2
MB 5	36	336	2.5	2.9
MB 6	33	256	<2.0	10

[a] ND, not determined.
[b] MB, mixed-breed dogs naturally infected with *Leishmania* parasites. Beagle dogs 919, 154, 155, and 158 were from the group of nonendemic controls.

TABLE 3. DTH skin reaction to leishmanin from the Barajas colony of beagles

Group and dog	Leishmanin[a] (mm)
Control uninfected	
7	<5.0
39	<5.0
64	<5.0
66	<5.0
68	<5.0
Infected	
Asymptomatic	
2	5.5
12	9.2
16	5.6
20	8.5
23	<5.0
28	11.8
44	7.0
Symptomatic	
14	<5.0
17	<5.0
19	5.5

[a] Values of induration or redness greater than 5 mm are considered positive reactions.

purified protein derivative, saline solution, and leishmanin diluent, which in all cases were skin test negative (data not shown).

IL-2, IL-6, and TNF production by PBMC. Supernatants of LSA-stimulated PBMC from asymptomatic dogs expressed a higher IL-2 activity compared with those in uninfected and symptomatic dogs (Fig. 1). The IL-2 activity in the latter group of animals was significantly ($P < 0.05$) lower than those in the control and asymptomatic animals. Of the symptomatic dogs, three of eight showed a complete abrogation of IL-2 activity.

It was also noted that a higher activity of TNF was found in supernatants of ConA-stimulated PBMC from asymptomatic dogs. The TNF activity was significantly ($P < 0.05$) decreased in supernatants of symptomatic dogs compared with those in the uninfected and asymptomatic animals (Fig. 2). A complete abrogation of TNF activity was observed in three of six symptomatic dogs. TNF activity in supernatants from LSA-stimulated PBMC was not detectable with the present bioassay.

Unlike the differences in IL-2 and TNF activity observed among the three distinct groups of dogs studied here, the IL-6 activity of LSA-stimulated PBMC supernatants from asymptomatic dogs did not differ significantly from those of uninfected and symptomatic animals (Fig. 3).

DISCUSSION

The present study was initiated on the observation that dogs from a group of naive beagles with primary *L. infantum* infection reacted mainly in two distinct ways: dogs either (i) presented clinically with VL or (ii) remained asymptomatic. This observation suggested that the two responses are mediated by different mechanisms and analysis of these phenomena might lead to an understanding of innate and possibly also acquired resistance or susceptibility to *L. infantum* in the dog.

We observed clear differences of both humoral and cellular immune responses between animals either resistant or susceptible to *L. infantum*. These differences were indicative of different regulatory mechanisms in the two groups of dogs studied.

The strong proliferative responses to leishmanial antigens in asymptomatic dogs corroborate findings with humans and mice indicating that in these species, immunity to VL is T cell mediated. In contrast, susceptible dogs, as shown previously also in human and mouse studies (14, 30, 32), failed to respond in vitro to LSA. This unresponsiveness appears to be antigen specific, as the lymphocytes from the same animals responded positively to both the mitogen ConA and to the Dohyvac vaccine preparation, an antigen unrelated to *L. infantum*. Moreover, not only can the naturally infected dogs be divided into resistant and susceptible animals, but their lymphocytes also exhibited, respectively, an enhanced and a depressed response to LSA. The latter finding seems to support the general validity of the data obtained from our *L. infantum* infection dog model studies. Thus, taken together, the present proliferative data suggest the occurrence in *L. infantum*-infected dogs of two types of lymphocyte function that correlate with either resistance or susceptibility to *L. infantum*.

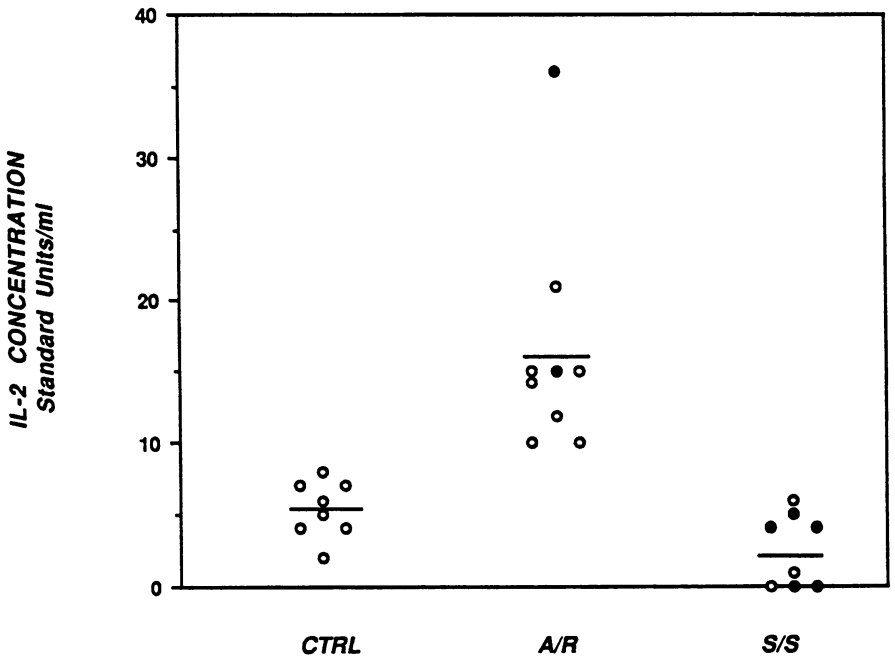

FIG. 1. IL-2 production after stimulation with *Leishmania* antigen by canine PBMC from infected asymptomatic or resistant (A/R), symptomatic or susceptible (S/S), and uninfected control (CTRL) animals. ○, PBMC from the Barajas colony of beagles; ●, PBMC from mixed-breed dogs naturally infected; ———, mean value.

The use of leishmanin in skin test experiments permitted us to obtain an indication of the T-cell function in vivo in the dogs studied. Enhanced DTH reaction to leishmanin was demonstrated mainly in asymptomatic dogs, indicating a difference in effector or regulatory T cells compared with that in dogs that develop leishmaniasis after infection. In this respect, it is noteworthy that the asymptomatic dogs appear to have antigen-specific lymphocyte proliferation in the absence of antileishmanial antibodies. This is exemplified by the fact that only one of eight asymptomatic dogs had a positive antileishmanial antibody titer (IFA titer, ≥1:80). Conversely, the experimentally infected dogs that developed

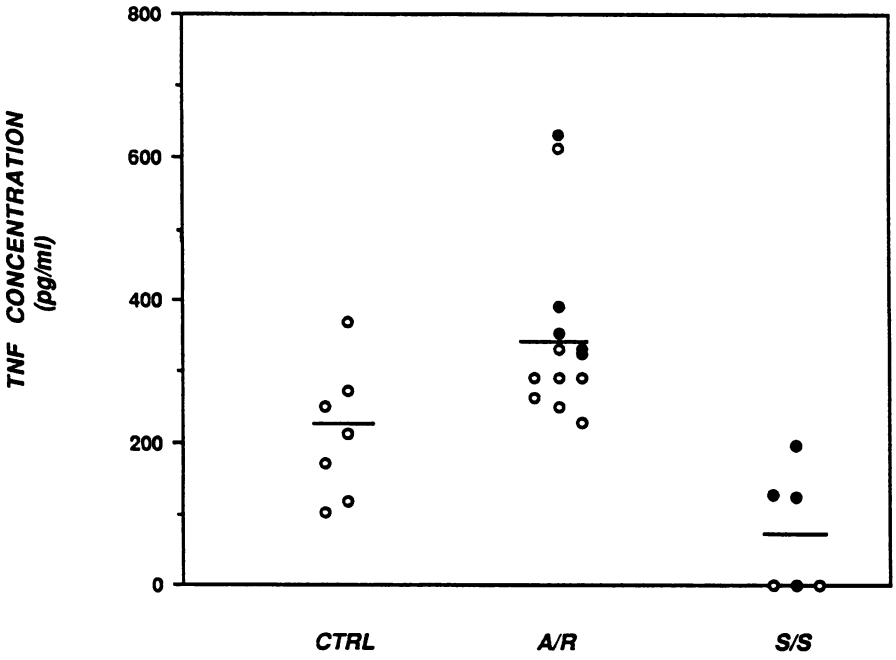

FIG. 2. TNF production by PBMC from infected asymptomatic or resistant (A/R), symptomatic or susceptible (S/S), and uninfected control (CTRL) animals after stimulation with ConA. Symbols are described in the legend to Fig. 1.

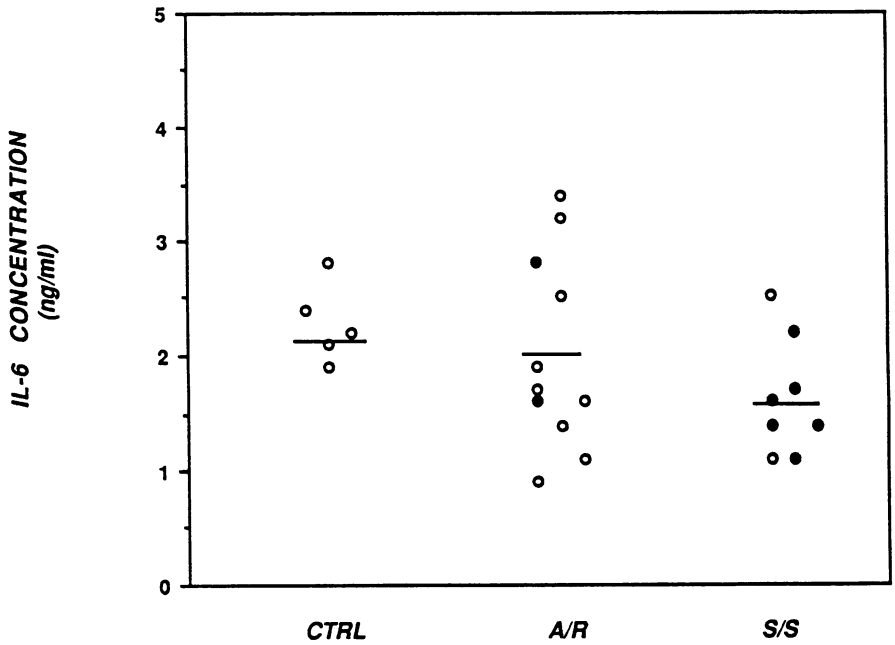

FIG. 3. IL-6 production after *Leishmania* antigen stimulation of PBMC from infected asymptomatic or resistant (A/R), symptomatic or susceptible (S/S), and uninfected control (CTRL) animals. Symbols are described in the legend to Fig. 1.

clinical signs of disease produced high serum antileishmanial antibodies (IFA titer, ≥1:320), but their lymphocytes failed to proliferate in vitro to LSA. Interestingly, the findings described here are in agreement with recent studies (5) reporting antigen-specific proliferative response of PBMC from asymptomatic dogs following natural *L. infantum* infections in the absence of antileishmanial antibodies.

Studies with murine *Leishmania* infection models indicate that resistance and susceptibility to *Leishmania major* is associated with the development of strong Th1 and Th2 responses, respectively (20, 31). These studies demonstrate that Th1 cells produce gamma interferon, TNF-α, and IL-2, promoting a DTH reaction and activation of macrophages to subsequently kill *Leishmania* parasites, whereas Th2 response is associated with increased production of IL-4, IL-5, IL-6, and IL-10, which inhibit macrophage activation (7, 25, 33). Our preliminary data on the production of cytokines in CVL suggest a possible role for IL-2 and TNF in resistance against *L. infantum* in both experimentally and naturally infected dogs. As regards in vitro IL-6 activity, there was no demonstrable association between the production of this cytokine and either resistance or disease development. The increased production of IL-6 has been suggested to be associated with higher levels of antibodies (16). However, in the present study no correlation was found between the high antibody levels observed in symptomatic animals and the IL-6 production. Although IL-2 and TNF are produced by Th1 cells and, similar to our findings, the production of these cytokines is associated with resistance, further studies including gamma interferon as well as other cytokines produced by Th2 cells such as IL-4, IL-5, and IL-10 must be carried out to determine whether a similar subpopulation of T cells is present in the dog and to study their possible role in this parasitic infection. The identification of the signals responsible for committing these cells to a particular profile of cytokine production is also of great importance, especially regarding vaccine development and treatment of such disease.

Further studies aimed at elucidating the immunological differences between the asymptomatic or resistant dogs and the symptomatic or susceptible dogs will address the following questions. (i) Can the differences in antibody levels observed between asymptomatic and susceptible animals be explained by differences in parasite load? (ii) Would animals recovered from the disease after drug treatment show a shift of the immunological parameters? This was suggested recently for individuals who have recovered from VL (18). (iii) Is the absence of DTH in susceptible dogs caused by trapping of antigen-specific lymphocytes in the lymphoid organs? This has been reported by Milon et al. (23), who showed that lymph nodes of susceptible mice harbor more *Leishmania*-specific $CD4^+$ T cells than those of resistant animals.

In summary, the present study of the immune status of dogs with experimental CVL involved determining antileishmanial antibody titers, antigen-specific proliferative responses, and DTH skin reactions as well as cytokine profiles. This approach permitted us to identify markers of disease progression for *L. infantum* infection in dogs. As the present research efforts focused on dogs without any clinical signs of leishmaniasis and dogs with established leishmaniasis, there may be differences with the field situation. A separate study which should also include dogs with intermediate stages of CVL would clarify this question. Nevertheless, we believe that monitoring and follow-up of such markers in areas where the disease is endemic will allow early diagnosis, and treatment could then be given when initial clinical symptoms of disease are present.

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